



## Factors involved in the maturation of murine dendritic cells transduced with adenoviral vector variants

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### Abstract

Adenoviral vector (Ad)-mediated gene transfer is an attractive method for manipulating the immunostimulatory properties of dendritic cells (DCs) for cancer immunotherapy. DCs treated with Ad have phenotype alterations (maturation) that facilitate T cell sensitization. We investigated the mechanisms of DC maturation with Ad transduction. Expression levels of a maturation marker (CD40) on DCs treated with conventional Ad, fiber-modified Ads (AdRGD, AdF35, AdF35ΔRGD), or a different serotype Ad (Ad35) were correlated with their transduction efficacy. The  $\alpha_v$ -integrin directional Ad, AdRGD, exhibited the most potent ability to enhance both foreign gene expression and CD40 expression, and induced secretion of interleukin-12, tumor necrosis factor- $\alpha$ , and interferon- $\alpha$  in DCs. The presence of a foreign gene expression cassette in AdRGD was not necessary for DC maturation. Maturation of DCs treated with AdRGD was suppressed by destruction of the Ad genome, inhibition of endocytosis, or endosome acidification, whereas proteasome inhibition increased CD40 expression levels on DCs. Moreover, inhibition of  $\alpha_v$ -integrin signal transduction and blockade of cytokine secretion affected the maturation of DCs treated with AdRGD only slightly or not at all, respectively. Thus, our data provide evidence that Ad-induced DC maturation is due to Ad invasion of the DCs, followed by nuclear transport of the Ad genome, and not to the expression of foreign genes.

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**Keywords:** Adenoviral vector; Dendritic cell; Maturation; CD40; Gene transduction

### Introduction

Dendritic cells (DCs) are unique in their ability to sensitize naive immune cells and to stimulate memory effector cells. Recent advances in DC biology, tumor immunology, and protein- and gene-transfer technology have made it possible to

manipulate DCs *ex vivo* to induce effective anti-tumor immune responses in cancer immunotherapy (Hsu et al., 1996; Nestle et al., 1998). Genetic modification of DCs with specific sequences of tumor-associated antigen (TAA) genes offers major advantages compared to other approaches. Following the transduction of TAA genes into DCs, full-length TAA proteins are expected to be physiologically processed and presented via major histocompatibility complex (MHC) class I molecules by DCs. Therefore, this is a method for activating specific anti-tumor CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) with the full spectrum of antigenic determinants. Furthermore, TAA gene-modified DCs also present MHC class II-restricted epitope(s) to

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CD4<sup>+</sup> T cells, and can express TAA continuously and MHC–peptide complexes for long periods (Yang et al., 1998; Sloan et al., 2002; Herrera et al., 2002).

Adenoviral vectors (Ads) were originally developed for gene therapy because of their high *in vitro* and *in vivo* transduction and expression efficacy in a broad range of cell types. Among various

techniques for transferring the TAA gene to DCs, the *ex vivo* engineering of DCs using Ad provides encouraging results (Wan et al., 1999; Kaplan et al., 1999; Steitz et al., 2001). The gene transduction efficiency of conventional Ad, which is based on the adenovirus subgroup C serotype 5 backbone, against mouse and human DCs is not sufficient, however, because DCs express little

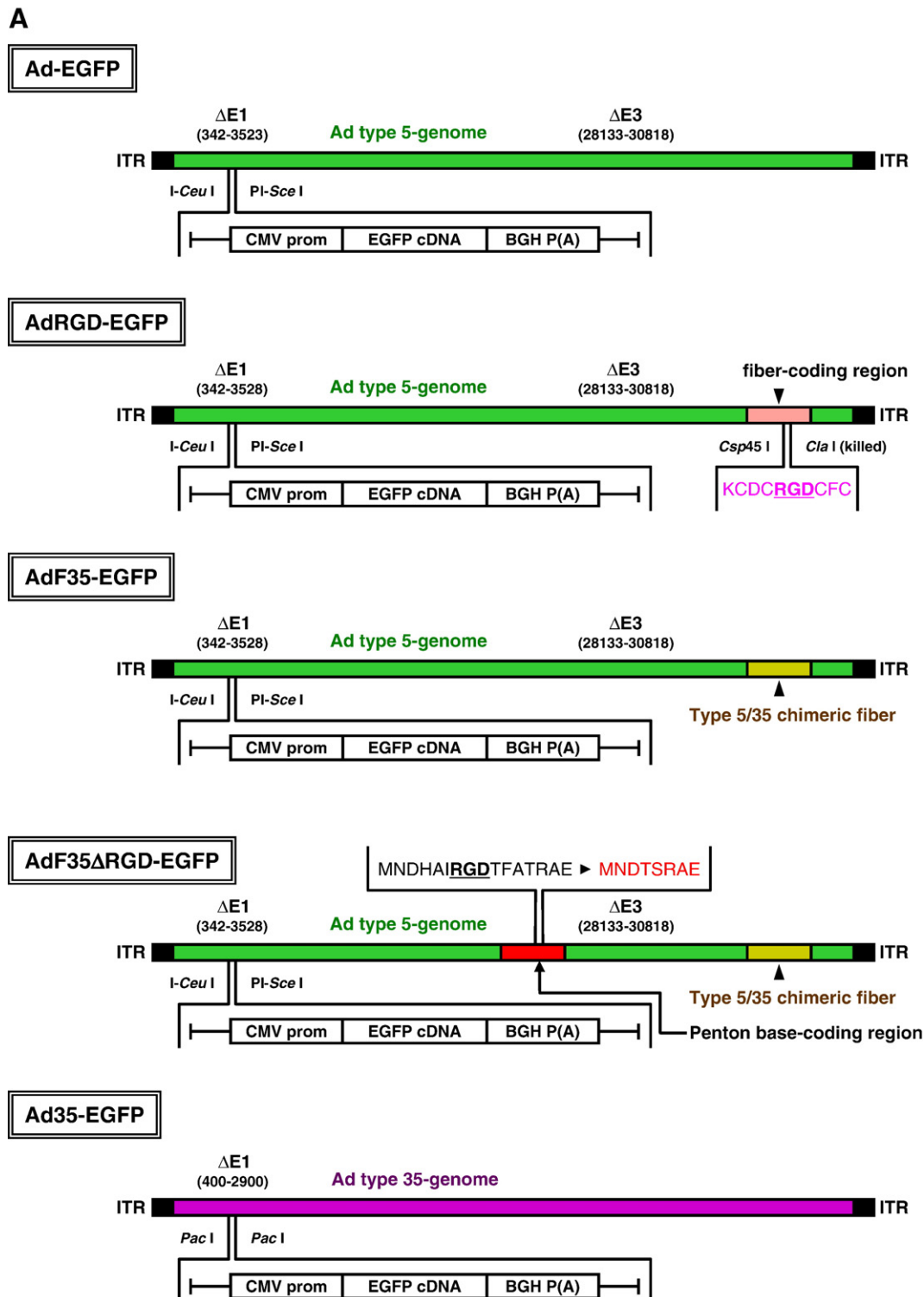


Fig. 1. Schematic representation of gene constructs (A) and tropism (B) of Ad variants used in this study. ITR, inverted terminal repeat; CMV prom, cytomegalovirus promoter; BGH P(A), bovine growth hormone polyadenylation signal.

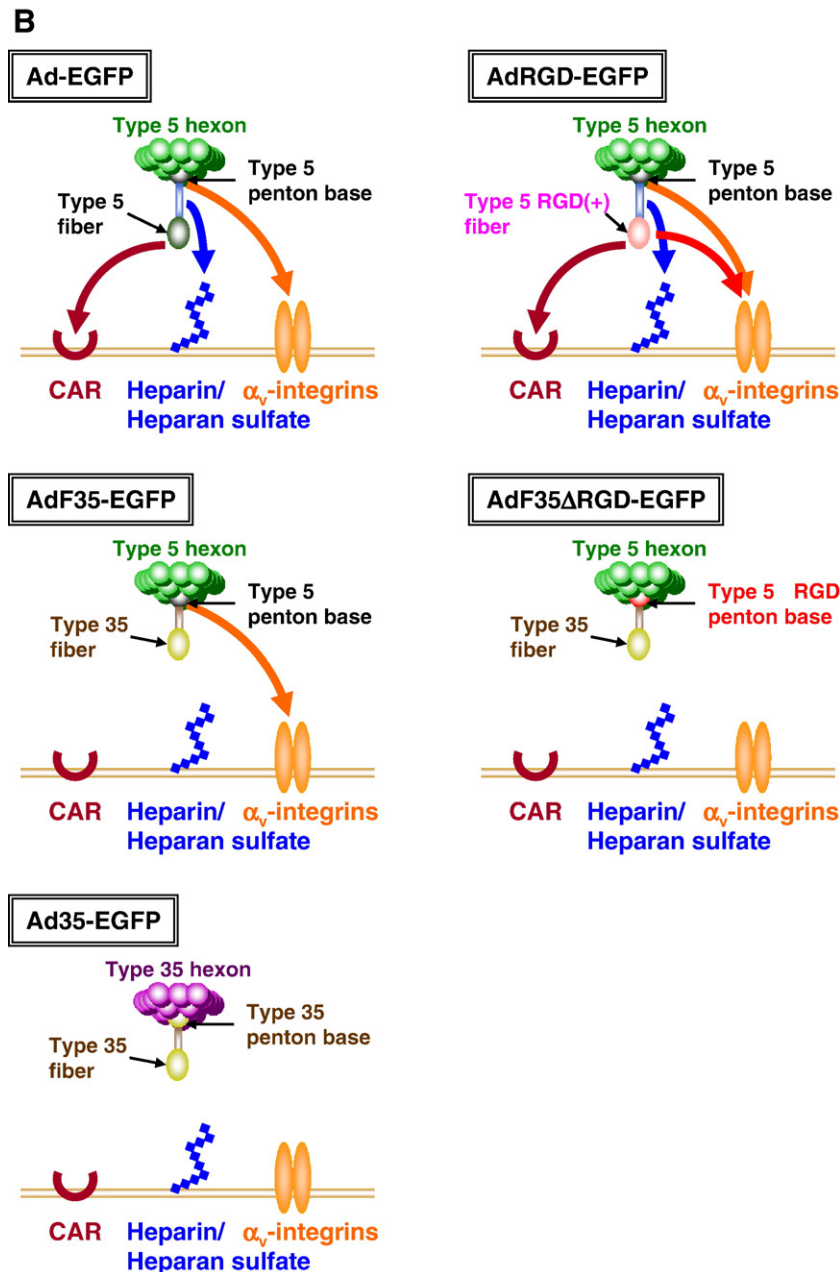


Fig. 1 (continued).

or no coxsackie–adenovirus receptor (CAR), which is the primary Ad receptor (Okada et al., 2001a,b, 2003a). We previously reported that the RGD fiber-mutant Ad (AdRGD), which targets  $\alpha_v$ -integrin upon attachment to cells by insertion of the RGD peptide to the HI loop of the fiber knob, exhibited highly efficient transduction compared to conventional Ads (Okada et al., 2001a,b, 2003a). AdRGD is a useful vector system that not only transfers the TAA gene (Okada et al., 2003b, 2006), but also confers a new function to DCs (Okada et al., 2005a,b) to potentially induce TAA-specific CTLs and anti-tumor efficacy in DC-based cancer immunotherapy. Our series of studies revealed that transduction with conventional Ad or AdRGD induced DC phenotype alterations (maturation), although the mechanism is unknown (Okada et al., 2001a,b, 2003a,b, 2005a,b, 2006).

The present study was aimed at elucidating the factors involved in the mechanisms underlying Ad-induced DC maturation using several Ad variants and inhibitors of the gene transduction pathway.

## Results

### *Relation between the transduction efficacy and DC maturation level*

We assessed the transduction efficacy and maturation level of DCs transduced with Ad variants by flow cytometric analysis. The gene constructs and tropism of Ad variants used in this study are summarized in Fig. 1. The DC maturation level was

determined by the CD40 expression level, because the increase in CD40 expression was remarkable as compared with the upregulation of other costimulatory molecules such as CD80 and CD86 (data not shown). An enhanced green fluorescent protein (EGFP) gene-carrying conventional Ad (Ad-EGFP), AdRGD (AdRGD-EGFP), and chimeric type 5/35 fiber-containing Ad (AdF35-EGFP) increased EGFP expression on DCs in a vector particle (VP)-dependent manner (Fig. 2 and Supplementary Fig. 1). Moreover, CD40 expression correlated with gene expression efficacy, and AdRGD exhibited the most potent ability for EGFP gene transduction and DC maturation induction. On the other hand, AdF35 $\Delta$ RGD-EGFP, which has type 35 fiber and no RGD motif in the penton base, and EGFP gene-carrying Ad type 35 (Ad35-EGFP), which differs from type 5 Ad in its mechanism of cell entry, induced little EGFP expression and DC maturation, indicating that type 35 Ad fiber recognizes CD46 (Gaggar et al., 2003), in which mouse DCs are defective (Sakurai et al., 2006). When DCs were transduced with the complex of Lipofectamine 2000 reagent (LF) and either AdF35 $\Delta$ RGD-EGFP or Ad35-EGFP, CD40 expression levels were slightly enhanced and had improved transduction efficacy. Similarly, LF-combined Ad-EGFP or AdF35-EGFP induced EGFP expression and DC

maturation more efficiently than each alone. Gene transduction efficacy and maturation level were decreased by AdRGD-EGFP in combination with LF, suggesting that LF might induce steric hindrance in the  $\alpha_v$ -integrin recognition by the RGD motif in the fiber knob of AdRGD. These data indicated that the Ad entry into DCs was a critical event for maturation.

We then evaluated the profiles of cytokine secretion in DCs treated with AdRGD-EGFP, which most efficiently promoted CD40 expression levels. Protein expression levels of interleukin-12 p40 (IL-12p40), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon- $\alpha$  (IFN- $\alpha$ ) in DCs increased with time after treatment with AdRGD, whereas IFN- $\alpha$  secretion was detected only at 24 h after transduction (Fig. 3). This finding suggested that the autocrine or paracrine effects of these cytokines were not the initial trigger for DC maturation by AdRGD transfection, although they might contribute to promote maturation.

#### *The effect of gene delivery into the nucleus and foreign gene expression by invading AdRGDs on DC maturation*

To elucidate the necessity of delivery of the Ad genome into the nucleus and foreign gene expression by invading AdRGDs

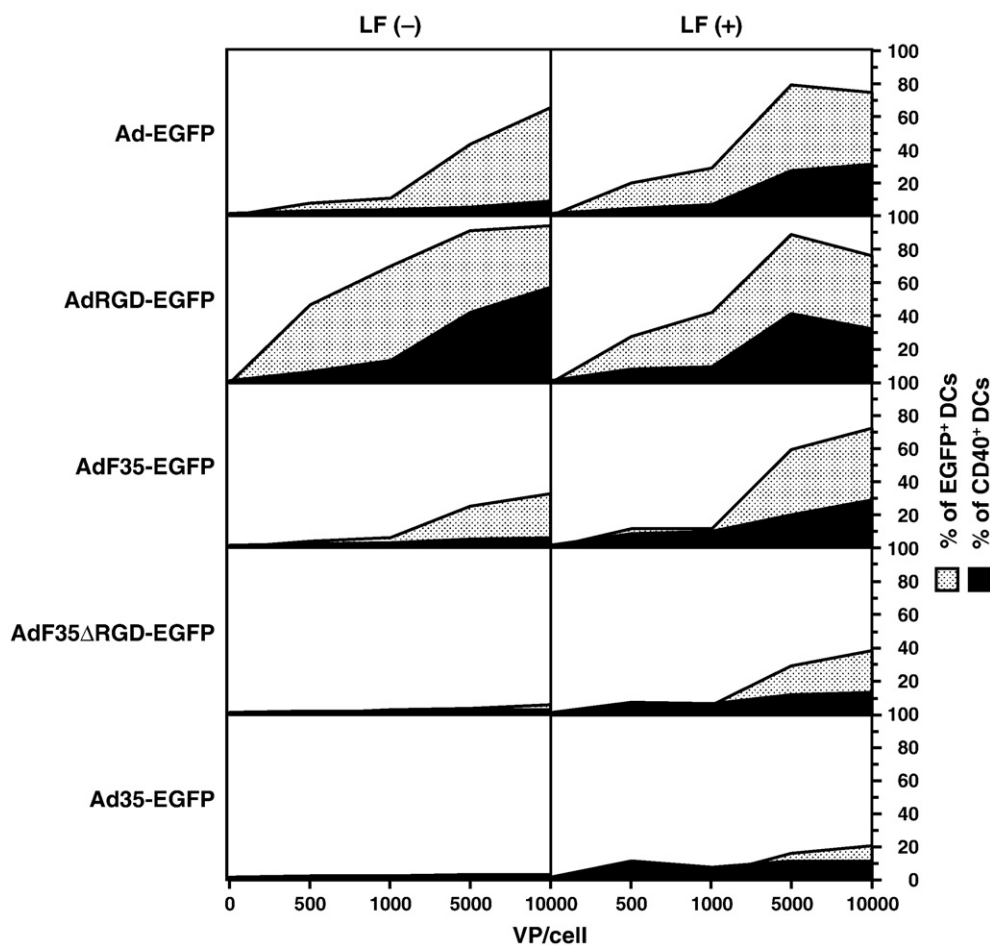


Fig. 2. EGFP gene transduction efficiency and the upregulation of CD40 expression in DCs transfected with Ad variants. DCs were transfected with each Ad variant alone or LF-Ad variant complexes, which were prepared by incubation of each Ad variant and LF (20  $\mu$ g/ml) for 20 min at room temperature, at the indicated VP/cell. After 24-h cultivation, these transduced DCs were stained by indirect immunofluorescence with anti-CD40 mAbs. Frequencies of EGFP gene-expressing DCs and CD40-positive DCs were analyzed by flow cytometry. Data are expressed as the mean of three independent experiments. Dot plot data are shown in Supplementary Fig. 1.

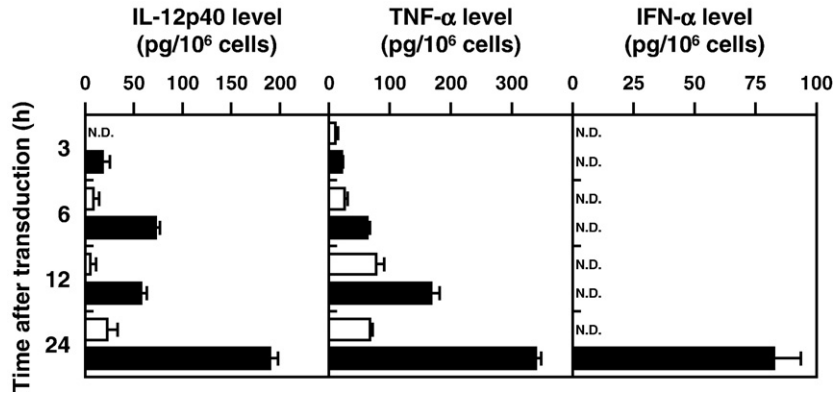


Fig. 3. Cytokine secretion profile in DCs transfected with AdRGD-EGFP. DCs were transfected with AdRGD-EGFP at 2000 VP/cell, and then cultured for the indicated time. Culture supernatants of mock DCs (open column) and transduced DCs (filled column) were harvested, and the concentrations of murine IL-12p40, TNF- $\alpha$ , and IFN- $\alpha$  proteins were measured by enzyme-linked immunosorbent assay. The data are presented as mean  $\pm$  SD of three independent cultures. N.D., not detectable.

for DC maturation, we prepared ultraviolet (UV)-irradiated AdRGD-EGFP (UV/AdRGD-EGFP) in which the Ad genome was destroyed. UV/AdRGD-EGFP maintained the ability to enter into the DCs (Fig. 4A). Cy3-labeled UV/AdRGD-EGFP bound to the DC plasma membrane 30 min after treatment, and then invaded the cytoplasm and eventually reached the nucleus, the same as Cy3-labeled AdRGD-EGFP. DCs treated with UV/AdRGD-EGFP did not express EGFP and did not exhibit

an increased frequency of CD40-positive DCs (Fig. 4B). On the other hand, AdRGD-Null, which carries no foreign gene expression cassette, induced DC maturation as well as AdRGD-EGFP. Noninfective heat-inactivated AdRGD-EGFP (Heat/AdRGD-EGFP), in which capsid proteins were denatured, did not induce either EGFP expression or DC maturation. These findings led us to conclude that DC maturation by AdRGD transfection was related to Ad genome delivery into the nucleus

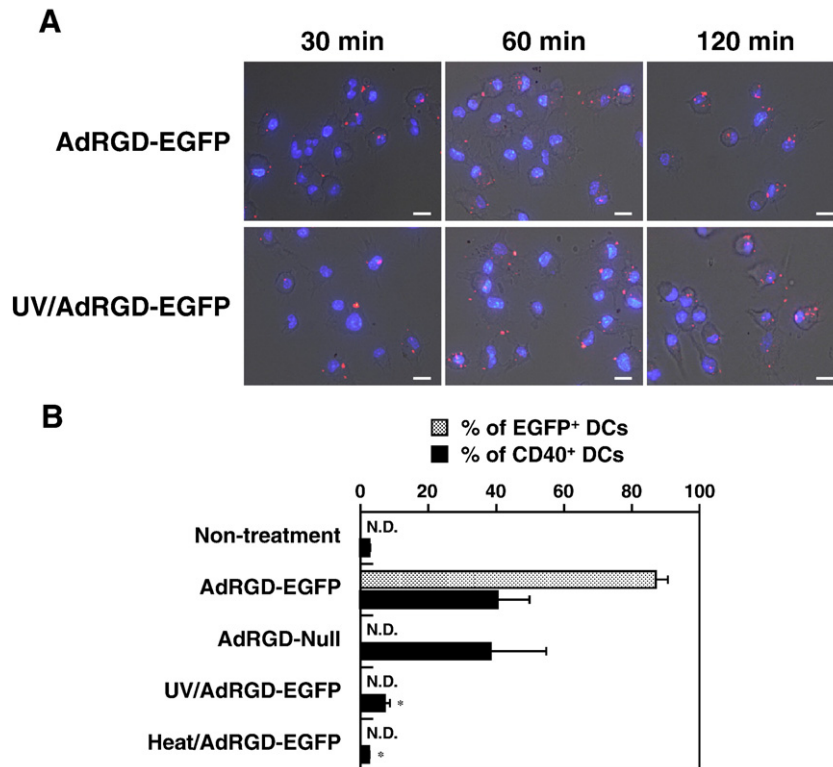


Fig. 4. Maturation frequency of DCs treated with inactivated AdRGD-EGFP or AdRGD-Null. (A); DCs were treated with Cy3-conjugated AdRGD-EGFP or UV/AdRGD-EGFP at 2000 VP/cell for 30, 60, or 120 min. Cellular uptake and intracellular trafficking of Cy3-labeled virions in DCs were observed under a fluorescence microscope. Nuclei were stained with Hoechst 33342. Scale bar, 10  $\mu$ m. (B); DCs were transfected with AdRGD-EGFP, AdRGD-Null, UV/AdRGD-EGFP, or Heat/AdRGD-EGFP at 2000 VP/cell. After 24-h cultivation, these transduced DCs were stained by indirect immunofluorescence with anti-CD40 mAbs. Frequencies of EGFP-positive DCs (dotted column) and CD40-positive DCs (filled column) were analyzed by flow cytometry. The data are presented as mean  $\pm$  SD of four independent cultures. Statistical analysis was performed using ANOVA, \* $p$  < 0.01, versus % of CD40<sup>+</sup> DCs treated with AdRGD-EGFP. N.D., not detectable.



by AdRGD particles invading the DCs, but not to foreign gene expression or capsid protein recognition in the DCs.

Effects of various Ad transduction pathway inhibitors on DC maturation

We analyzed the effect of intracellular traffic of Ad particles on DC maturation using various inhibitors. Because AdRGD most efficiently induced DC maturation, we predicted that DC maturation was involved in the signal transduction through  $\alpha_v$ -integrin binding of the RGD motif on the Ad-penton base. We investigated the level of DC maturation induced by AdRGD in the presence of wortmannin (WM), a phosphoinositide-3-OH kinase (PI3K) inhibitor that is activated by  $\alpha_v$ -integrin binding. WM slightly reduced the frequency of EGFP-positive and CD40-positive DCs treated with AdRGD-EGFP (Fig. 5). This result indicated that the  $\alpha_v$ -integrin–RGD motif interaction was slightly related to the upregulation of CD40 expression on the DCs. AdRGD entry into the cells through the endocytosis pathway is dependent on integrin. The transduction efficiency

and maturation level of DCs treated with AdRGD-EGFP were slightly decreased in the presence of cytochalasin B (CB), an endocytosis inhibitor. DC maturation induced by AdRGD required Ad particles to enter into cells, but signal transduction followed by endocytosis activity contributed little to DC maturation. Subsequently, we investigated the necessity of endosome escape, which indicates the transition of Ad particles into the cytoplasm, for induction of the DC maturation signal using chloroquine (CQ), which inhibits the change of acidity in the endosome. CQ clearly decreased the expression level of both the foreign gene and CD40 on the DCs. Therefore, these results indicated that DC maturation required Ad entry into the cytoplasm and the delivery of the Ad genome into the nucleus. Lactacystin (LC), a proteasome inhibitor, greatly enhanced the EGFP and CD40 expression levels in DCs treated with AdRGD, suggesting that LC might increase the number of Ad particles and Ad genomes by inhibiting Ad degradation. This finding supported the hypothesis that DC maturation is related to increased delivery of the Ad genome to the nucleus. In addition, the CD40 and EGFP expression levels of DCs treated with AdRGD in the presence of brefeldin A (BA), which blocks cytokine secretion from DCs, were slightly higher than those of AdRGD-transduced DCs without inhibitors. We again confirmed that cytokine (*i.e.*, IL-12, TNF- $\alpha$ , and IFN- $\alpha$ ) secretion from the DCs did not act as the initial trigger for DC maturation.

Together, these results demonstrated that DC maturation induced by AdRGD required AdRGD entry into the cytoplasm through endocytosis, and that the trigger for DC maturation functioned after AdRGD escaped the endosome.

Discussion

In Ad-mediated gene transduction, Ad first attaches to the cell through an interaction between the carboxy terminus of the fiber knob of the Ad particle and a cell surface receptor, known as the CAR (Bergelson et al., 1997; Roelvink et al., 1998, 1999). Following the high-affinity binding to CAR, a lower-affinity interaction between  $\alpha_v\beta_3$ - or  $\alpha_v\beta_5$ -integrins and the RGD motif in the Ad-penton base facilitates internalization of the virion by receptor-mediated endocytosis (Wickham et al., 1993, 1994; Mathias et al., 1994). After internalization, Ad penetrates the cytoplasm, which involves an acid-dependent conformational change in the Ad-penton base (Wang et al., 2000), and the recombinant viral DNA is delivered to the nucleus. We and others previously reported that Ad transduction induces DC maturation (Okada et al., 2001a, 2003a; Hirschowitz et al., 2000; Morelli et al., 2000; Rea et al., 1999; Philpott et al., 2004; Liu et al., 2003; Molinier-Frenkel et al., 2003), although the details of the mechanism were unknown. In the present study, we attempted to elucidate the factors involved in Ad-mediated DC maturation by examining the Ad transduction pathway.

We first determined that the gene transduction efficiency of Ad variants closely correlated with DC maturation levels, and that AdRGD was the most efficient vector for DC maturation among five Ad variants. In addition, our results using LF-Ad variant complexes indicated that DC maturation required

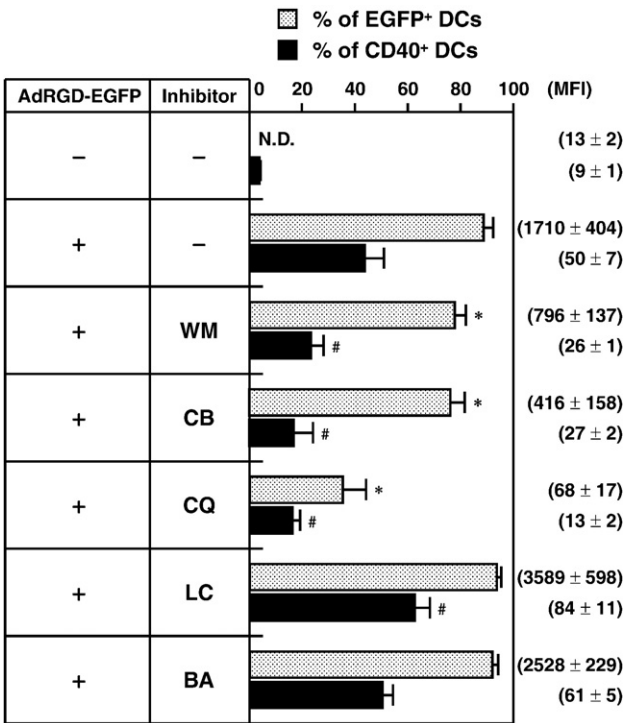


Fig. 5. Effect of various inhibitors on EGFP gene transduction efficiency and CD40-positive frequency in DCs transfected with AdRGD-EGFP. DCs were preincubated for 1 h with 100 nM wortmannin (WM), 20  $\mu$ M cytochalasin B (CB), 300  $\mu$ M chloroquine (CQ), 10  $\mu$ M lactacystin (LC), or 5  $\mu$ g/ml brefeldin A (BA), and then transfected with AdRGD-EGFP at 2000 VP/cell in the presence of each inhibitor. After 24-h cultivation, these transduced DCs were stained by indirect immunofluorescence with anti-CD40 mAbs. Frequencies of EGFP-positive DCs (dotted column) and CD40-positive DCs (filled column) were analyzed by flow cytometry. The data are presented as the mean  $\pm$  SD of four independent cultures. Statistical analysis was performed using ANOVA, \* $p$  < 0.01, versus % EGFP+ DCs treated with AdRGD-EGFP without inhibitors, and # $p$  < 0.01, versus % CD40+ DCs treated with AdRGD-EGFP without inhibitors. N.D., not detectable. The numerical value in parentheses indicates mean fluorescence intensity (MFI) of EGFP or CD40 expression.

foreign gene expression based on the entry of Ad particles into the cells, regardless of receptor-dependent or -independent endocytosis. On the other hand, heat-inactive AdRGD did not induce DC maturation as well as noninfective Ad treated with sonication or surfactant (data not shown), suggesting that pulsation of the Ad capsid proteins was not related to DC maturation. Although several groups have argued that DC maturation is mediated by Ad component proteins, *e.g.*, the penton base or fiber knob domain (Liu et al., 2003; Molinier-Frenkel et al., 2003), our data agreed with the report that DC maturation is independent of fiber content (Schoggins et al., 2005).

The necessity of AdRGD particle entry via endocytosis for DC maturation was also supported by an experiment using an endocytosis inhibitor. Thus, we examined the relationship between an endocytosis signal and DC maturation. The interaction between the RGD motif and  $\alpha_v$ -integrins, an endocytosis receptor, induces the activation of PI3K (Li et al., 1998; Nemerow, 2000). Previous reports showed that the nuclear factor- $\kappa$ B signal mediated by the integrin–PI3K pathway induces DC maturation through TNF- $\alpha$  production (Philpott et al., 2004). Our result from an experiment using a PI3K inhibitor, however, indicated that the  $\alpha_v$ -integrin–PI3K pathway was not mainly, but rather only partially, involved in DC maturation induced by AdRGD transduction. Moreover, our collaborators confirmed that the toll-like receptor 9, which recognizes microbial invasion in endosomes, and its adaptor molecule (MyD88) do not contribute to Ad-mediated DC maturation (Yamaguchi et al., 2007).

Additionally, AdRGD-mediated DC maturation was associated with endosome escape by the AdRGD particles, and a proteasome inhibitor promoted both foreign gene expression and DC maturation. These results strongly suggest that DC maturation levels reflect the amount and stability of the AdRGD particles in the cytoplasm that delivered the recombinant viral genome into the nucleus. Moreover, DC maturation levels were equal between AdRGD-EGFP- and AdRGD-Null-treated DCs, indicating that AdRGD induction of DC maturation was not influenced by the presence of the foreign gene expression cassette. This idea is supported by previous reports demonstrating that Ad-mediated DC maturation is independent of gene expression (Hirschowitz et al., 2000; Morelli et al., 2000; Rea et al., 1999). On the other hand, even if AdRGD particles were able to enter into the DCs, destruction of the viral genome by UV irradiation (Mulders et al., 1998) did not induce DC maturation. Therefore, we concluded that delivery of the recombinant viral genome into the nucleus, but not foreign gene expression, was essential for DC maturation.

Although DCs transduced with AdRGD secreted inflammatory cytokines such as IL-12, TNF- $\alpha$ , and IFN- $\alpha$ , blocking cytokine secretion did not reduce the DC maturation levels. This result was consistent with a previous report indicating that Ad entry, but not the secretion of TNF- $\alpha$  or IFN- $\alpha$  through the nuclear factor- $\kappa$ B pathway, triggered the DC maturation (Seiler et al., 2007). We do not think that the autocrine and paracrine effects of these cytokines triggered DC maturation in AdRGD transduction, but speculate that they might at least contribute to

promote and maintain the DC maturation status (Hensley et al., 2005).

In conclusion, our data provide evidence that DC maturation with Ad-mediated transduction results from the delivery of the Ad genome into the nucleus. Our results also suggest that the nucleus has certain molecular mechanisms for recognizing the Ad genome. A recent report suggests that recognition of the Ad particles occurs chiefly via a toll-like receptor-independent nucleic acid-sensing mechanism that recognizes the viral dsDNA genome, and that delivery of the viral genome triggers IFN regulatory factor 3 phosphorylation (Nociari et al., 2007). Further studies to analyze more detailed events in the nucleus will allow us to better understand the factors that contribute to the efficiency and safety of Ad gene therapy and DC-based immunotherapy.

## Materials and methods

### Mice

Female C57BL/6 mice (H-2<sup>b</sup>), 8 to 12 weeks of age, were purchased from Japan SLC Inc. (Hamamatsu, Japan), and were maintained under specific pathogen-free conditions. Animals were handled in accordance with the Osaka University and Kyoto Pharmaceutical University guidelines for the welfare of experimental animals.

### Vectors

Ad-EGFP (Okada et al., 2001b), AdRGD-EGFP (Okada et al., 2001b), and AdF35-EGFP (Mizuguchi and Hayakawa, 2002; Sakurai et al., 2003) were previously constructed by an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). AdRGD-Null, which is identical to AdRGD but without the gene expression cassette, was previously constructed (Okada et al., 2004). AdF35 $\Delta$ RGD-EGFP was constructed using the vector plasmid pAdHM35 as follows. pAd22 (Mizuguchi et al., 2002), which has the *Pme* I–*Asc* I fragment of the adenovirus genome (bp 13255–15672), was digested with *Asc* I and *Hinc* II and ligated with *Asc* I/*Hinc* II-digested pHM5.1, which is a derivative of pHM5 (Mizuguchi and Kay, 1999), resulting in pAd23, which contains the *Hinc* II–*Asc* I fragment of the Ad genome (bp 14383–15672). pAd23 was then digested with *Hinc* II and *Eag* I and ligated with *Hinc* II/*Eag* I-digested pAd25 $\Delta$  (Mizuguchi et al., 2002), which contains the *Hinc* II–*Eag* I fragment of the Ad genome (bp 14383–15229) with a change in the *Sac* II–*Bss*H II fragment (bp 15133–15209) into oligonucleotide 4 (5'-GGCAATGCAGCCGGTGGAGGACATGAACGATACATCTAGAGCTGAGGAGAAG-3') and oligonucleotide 5 (5'-CGCCGTTACGTCGGCCACCTCCTGTACTTGCTATGTAGATCTCGACTCCTCTTCGCGC) (the *Xba* I site is underlined and the RGD peptide motif of the penton base, MNDHAIRGDT-FATRAE, was changed to MNDTSRE), resulting in pAd26 $\Delta$ . The altered sequence containing the penton base was then introduced into pAdHM34 (Mizuguchi and Hayakawa, 2002) by continuous routine plasmid construction using *in vitro* ligation. The resulting vector plasmid pAdHM35 had a complete E1/E3-

deleted Ad genome with I-Ceu I, Sma I, and PstI sites in the E1 deletion region, chimeric type 5/35 fiber sequence, and the deletion of the RGD peptide motif of the penton base. The EGFP expression cassette was inserted in the E1 deletion region by an improved *in vitro* ligation method, resulting in AdF35ΔRGD-EGFP. Ad35-EGFP, which was based on the adenovirus serotype 35 backbone with deletion of the E1 region, was generated as previously described (Sakurai et al., 2003). All recombinant vectors were propagated, purified by two rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at  $-80^{\circ}\text{C}$ . Vector particle (VP) titers were evaluated spectrophotometrically using the method of Maizel et al. (1968). Gene constructs and tropism of Ad variants are summarized in Fig. 1.

#### *Generation of mouse bone marrow-derived DCs*

DCs were prepared according to the method of Lutz et al. (1999) with a slight modification. Briefly, bone marrow cells flushed from the femurs and tibias of C57BL/6 mice were seeded at  $5 \times 10^6$  cells per sterile 100-mm bacterial grade culture dish in 10 ml of RPMI 1640 medium containing 10% fetal bovine serum, 40 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF; kindly provided by KIRIN Brewery Co., LTD., Tokyo, Japan), 50  $\mu\text{M}$  2-mercaptoethanol, and antibiotics. On day 3, another 10 ml of the culture medium was added to the dish for medium replenishment. On day 6, 10 ml of the culture supernatant was collected and centrifuged at 1500 rpm for 5 min at room temperature, and the pellet was resuspended in 10 ml of fresh culture medium, and then returned to the original dish to conserve unattached cells. Eight-day old DCs (non-adherent cells) were harvested and used as intact immature DCs in subsequent experiments.

#### *Viral transduction into DCs*

DCs were suspended at a concentration of  $5 \times 10^6$  cells/ml in fetal bovine serum-free RPMI 1640 medium and placed in a 50-ml conical tube. Each Ad variant was added at various VP/cell, the suspension was mixed well, and the tube was incubated at  $37^{\circ}\text{C}$  for 2 h with occasional gentle agitation. In some cases, DCs were transfected with vector–cationic lipid complexes that were prepared by incubation of each Ad variant and 20  $\mu\text{g}/\text{ml}$  LF (Invitrogen, Carlsbad, CA) at room temperature for 20 min. The cells were washed three times with phosphate-buffered saline (PBS) and resuspended in a suitable solution.

#### *Analysis of EGFP gene transduction efficiency and CD40 expression levels in transduced DCs*

The frequencies of EGFP-positive and CD40-positive transduced DCs cultured in GM-CSF-free medium for 24 h post-transfection were assessed by flow cytometric analysis. Briefly,  $10^6$  cells in 100  $\mu\text{l}$  staining buffer (PBS containing 0.1% bovine serum albumin and 0.01%  $\text{NaN}_3$ ) were incubated for 30 min on ice with the anti-Fc $\gamma$ R2/3 monoclonal antibody (mAb; clone 2.4G2) to block nonspecific binding of the

subsequently used immunoreagents. The cells were incubated for 30 min on ice using the manufacturer's recommended amounts of biotinylated anti-CD40 mAb (clone GL1). The cells were then resuspended in 100  $\mu\text{l}$  staining buffer containing PerCP-conjugated streptavidin at a 1:200 dilution, and non-specific binding was measured using PerCP-conjugated streptavidin alone. After incubation for 30 min on ice, EGFP gene transduction efficiency and the CD40 expression levels in the transduced DCs were analyzed by flow cytometry on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Tokyo, Japan), acquiring 30,000 events by forward and side scatter gating to exclude cell debris. Between all incubation steps, cells were washed three times with staining buffer. All immunoreagents used in the present study were purchased from BD Bioscience (San Jose, CA).

#### *Analysis of cytokine production in transduced DCs*

DCs transduced with AdRGD-EGFP at 2000 VP/cell and mock DCs were cultured in GM-CSF-free culture medium for 3, 6, 12, or 24 h. The culture supernatants were collected, and the IL-12p40, TNF- $\alpha$ , and IFN- $\alpha$  concentrations were measured with the corresponding enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, CA).

#### *Preparation of inactivated AdRGD-EGFP*

UV-irradiated AdRGD-EGFP (UV/AdRGD-EGFP) was prepared according to the methods reported by Kodama et al. (2001) and Cotton et al. (1992). Heat/AdRGD-EGFP was prepared by incubation in a hot bath for 5 min.

#### *Analysis of cellular uptake and intracellular trafficking of UV/AdRGD-EGFP in DCs*

AdRGD-EGFP and UV/AdRGD-EGFP were labeled by Cy3 (Amersham Pharmacia Biotech, Tokyo, Japan) according to the method of Miyazawa et al. (1999). The Cy3-labeling efficiency, the ratio of Cy3-fluorescence intensity to the number of virions, was equal for both vectors (data not shown). Cy3-labeled AdRGD-EGFP or UV/AdRGD-EGFP was added to cultured DCs at 2000 VP/cell. After 30, 60, or 120 min, DCs were repeatedly washed with PBS and treated with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) for nuclear staining. Cellular uptake and intracellular trafficking of Cy3-labeled virions were observed under a fluorescence microscope (BZ-8000; Keyence Corporation, Osaka, Japan).

#### *Treatment with various inhibitors in the transfection procedure*

DCs were preincubated with 100 nM WM (Sigma Chemical Co., St. Louis, MO), 20  $\mu\text{M}$  CB (Sigma Chemical Co.), 300  $\mu\text{M}$  CQ (Sigma Chemical Co.), 10  $\mu\text{M}$  LC (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), or 5  $\mu\text{g}/\text{ml}$  BA (Sigma Chemical Co.) for 1 h, and then transfected with AdRGD-EGFP at 2000 VP/cell in the presence of each inhibitor. We confirmed that the inhibitors had no effect on cell



viability at the concentrations used in this experiment (data not shown).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.12.043.

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